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Isolation, expression and biochemical characterization of recombinant hyoscyamine-6 β -hydroxylase from *Brugmansia sanguinea* – tuning the scopolamine production†

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Hyoscyamine-6 β -hydroxylase (H6H, EC 1.14.11.11) is a plant enzyme that catalyses the last two steps in the biosynthesis of the anticholinergic drug scopolamine, *i.e.* the hydroxylation of hyoscyamine to 6 β -hydroxyhyoscyamine (anisodamine) and subsequent oxidative ring-closure to the 6,7- β -epoxide. A H6H gene homologue was isolated from the plant *Brugmansia sanguinea* (BsH6H) and recombinantly cloned into *Escherichia coli*, expressed and purified using an effective SUMO-fusion procedure. Enzymatic activity is approximately 40-fold higher for the first reaction step and the substrate affinity is comparable to other characterized H6H homologues ($K_m \sim 60 \mu\text{M}$). Truncation of an H6H enzyme flexible N-terminal region yields an active and stable yet more compact enzyme version.

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Introduction

Anisodamine, scopolamine and related tropane alkaloids (*e.g.* atropine: ($\pm$)-hyoscyamine) are valuable pharmaceutical compounds with anticholinergic activity¹ produced as self-defence agents in a variety of plants of the *Solanaceae* family. These alkaloids, which show remarkable hallucinogenic and sedative effects, are used for the treatment of motion sickness, gastrointestinal spasms and nausea. They are listed among the top compounds in the World Health Organization (WHO) essential drug catalogue, including atropine, ipratropium bromide, scopolamine butylbromide and tiotropium bromide.² Compared to the parent hyoscyamine (1, Scheme 1), scopolamine (3) possesses a remarkably higher pharmaceutical activity and has less toxic side-effects. Recently it has been shown that 6 β -hydroxyhyoscyamine (2) (anisodamine), the biosynthetic intermediate between hyoscyamine (1) and scopolamine (3), has the lowest neurotoxicity, suggesting this drug as new therapeutic agent against Alzheimer's disease and organophosphate poisoning.³ Despite their beneficial pharmaceutical properties and high therapeutic demand, the lack of high yielding and stereospecific total synthesis approaches, requires the production of 1–3 to rely on plant ex-

traction.^{1,4} Therefore the exploration of new reliable plant sources is of crucial interest.

The biosynthesis of the tropane scaffold starts from putrescine and L-arginine. It involves a series of different enzymes with hyoscyamine-6 β -hydroxylase (H6H) being responsible for the last two steps in this reaction cascade, *i.e.* the hydroxylation and epoxidation of hyoscyamine (Scheme 1).⁵ Among the different *Solanaceae* species, 6 β -hydroxyhyoscyamine (2) can be found in much higher quantities than scopolamine (3), which has been attributed both to a kinetically less favored epoxidation step and low expression levels.^{5,6} To overcome this low efficiency, several working groups have reported attempts to increase the expression level of the key enzyme H6H in various plant species.^{7–16} Another useful approach involves the recombinant cloning and

Scheme 1 H6H catalyzes the last two steps of the scopolamine biosynthesis.

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expression of H6H in *Escherichia coli*^{3,17} or *Saccharomyces cerevisiae*¹⁸ with subsequent *in vitro* transformation of **1** by the purified enzyme. In this regard, site-specific mutagenesis is able to enhance enzyme activity, but identifying “hot-spots” for targeted mutagenesis is presently random since structural information about H6H is very limited.⁸ Although combination of these approaches can boost the production of anisodamine and scopolamine somewhat, the epoxidation step still remains slow. Herein we present the cDNA isolation and expression of H6H from a new plant source, *Brugmansia sanguinea*, reported to be a good scopolamine producer,¹⁹ suggesting presence of a more active H6H. Using an efficient and easily removable SUMO-tag,²⁰ production of H6H recombinant enzyme was optimized to 25 mg L⁻¹ cell culture. The kinetic parameters of the hydroxylation as well as epoxidation step were determined.

Although the epoxidation of **2** is about 40-times slower than hydroxylation of **1** with the purified enzyme, preparations with *BsH6H*-plasmid bearing whole cells dramatically boost the scopolamine production, as has been recently indicated also for a different H6H homologue by another group.²¹

Results and discussion

Isolation and cloning of *H6H*cDNA

Since scopolamine content in plants is directly correlated to hyoscyamine-6 β -hydroxylase content and activity, engineering of this enzyme and the exploration of more potent H6H plant species in order to boost scopolamine yield has attracted considerable interest.^{7–18} *B. sanguinea* is a flowering shrub from the *Datureae* tribe, used in the Andes region as a potent hallucinogenic and sedative drug for various medicinal and ritual practices.²² It has been identified as a potent scopolamine producer based on plant part drug content analysis although closer genetic and biochemical details are missing.¹⁹ To isolate the H6H coding sequence from *B. sanguinea*, primers were designed using the reported sequence of *Brugmansia candida*.¹⁸ Using the primer set, we were able to isolate a H6H homologue with 1280 bp length from root cDNA. After verifying the sequence of the isolated gene, the gene was named as *BsH6H*. For the *in vitro* enzyme assay, *BsH6H* was overexpressed in *E. coli* BL21(DE3) fused to a small ubiquitin-like modifier protein (SUMO) bearing an N-terminal His₆-tag (His-SUMO-*BsH6H*). The SUMO-fusion protein strategy was chosen to increase protein overexpression and stability, and achieve a His-tagged free H6H protein after cleavage of the fusion tag.^{20,23}

Expression and purification of *BsH6H*

Using SUMO as an expression tag the overall yield of expressed fusion protein could be boosted to about 25 mg L⁻¹ cell culture (52.034 kDa). A second advantage of this expression system is that inclusion body formation is avoided and that the complete SUMO-fusion including the His₆-tag can be readily removed with SUMO protease (Fig. S1†), en-

abling biochemical characterization of *BsH6H* without interfering tag-effects. Final purification by gel filtration revealed that *BsH6H* predominantly exists as monomers (approx. 90%, Fig. 1), indicating the lack of surface exposed cysteine residues.

Biochemical characterization of *BsH6H*

The *BsH6H* protein consists of 343 amino acids, reflected by molecular weight of the monomer of 38.768 kDa after cleavage of the His₆-SUMO-tag (Fig. S1†) and a theoretical pI of 4.75. BLASTp analysis reveals highest sequence homology of *BsH6H* with the H6H homologue of *Brugmansia arborea* (97%, ALD59773) accounting for 10 amino acid alterations. High sequence identity is also observed with H6H homologues from *Atropa baetica* (92%, ABR15749), *Atropa belladonna* and *Anisodus acutangulus* (91%, BAA78340 and ABM74185, respectively, Fig. S2†). *BsH6H* possesses an α -ketoglutarate binding motif (H217-L230) and two iron ion binding motifs (C55-F68 and V63-V277, Fig. S2†). In accord with related H6H homologues, the motif His-X-His (H217, Asp219, His274) and two amino acid residues (Arg284, Ser286) that stabilize α -ketoglutarate are highly conserved. Overall, four amino acid alterations can be observed that are so far unprecedented throughout H6H homologues (*i.e.* K53 $\rightarrow$ E53, P69 $\rightarrow$ S69, R282 $\rightarrow$ S282, Y333 $\rightarrow$ F333). To obtain further structural insights the 3D-structure of *BsH6H* was modeled based on the closest PDB hit of *Arabidopsis thaliana* anthocyanidin synthase (Fig. 2, PDB: 1gp6, sequence identity 28%). Structural overlay and comparison with other H6H homologues reveals an overall Rossmann fold with a flexible loop between Lys91–Asp130 (Fig. 2). Furthermore, the N-terminal region (30 amino acids) of all H6H homologues indicates high flexibility including no clear secondary structural elements. This suggests that this region contributes no substantial stabilization for the function of the protein. Therefore, we designed a truncated H6H version from *A. belladonna*, missing 30 N-terminal amino acids in order to study the effect of an N-terminal shortening on the activity of the enzyme (*AbtH6H*).

Enzymatic activity of *BsH6H* and *AbtH6H*

Kinetics for the transformation of hyoscyamine **1** to the intermediate **2** and scopolamine **3** were accessed following a

Fig. 1 Protein FPLC (Sephadex G-100) of *BsH6H*-SUMO fusion and tag-free *BsH6H*, indicating the presence of monomers.

Fig. 2 Structure model of BsH6H (light blue) superimposed with the closest PDB hit anthocyanidin synthase (gray, PDB ID: 1gp6). The active site of anthocyanidin synthase is highlighted (red).

procedure published before.¹⁷ In order to evaluate the influence of the SUMO expression tag, we included both SUMO and SUMO-free versions of each protein (*BsH6H* and *AbtH6H*) in the kinetic analysis. After incubation of each substrate with H6H (0.6 μM) for 1 h, and subsequent EtOAc extraction and concentration, the extracted compounds were analyzed by LC-MS. Retention times on a RP-C18 column are 2.8 min (3), 2.9 min (2) and 3.2 min (1), respectively (Fig. S3†). Production of 6 β -hydroxyhyoscyamine and scopolamine obeys Michaelis–Menten kinetics within the first hour of reaction, enabling us to extract the kinetic parameters. *BsH6H* as well as *AbtH6H* are stable in 20 mM Tris buffer (pH 7.6) at -20°C . At 4°C both enzyme lose 50% of activity after 72 h. On closer examination, *BsH6H*'s affinity for 1 and 2 is comparable to related species,^{5,24,25} however, the epoxidation step occurs about 10-times faster than for the previously described *A. belladonna* homologue of this enzyme (Table 1).¹⁷ Truncation of the enzyme (*AbtH6H*) does not alter the kinetic performance, therefore reflecting an interesting opportunity to obtain a more compact enzyme with increased crystallization potential. In a similar fashion, the SUMO-tag also does not interfere with the activity of the enzyme. In accordance with

previous reports,^{5,8,21} we could confirm, that using whole cells in the fermentation process results in higher yields of scopolamine (3), whereas the intermediate 6 β -hydroxyhyoscyamine (2) predominantly accumulates if the purified H6H enzyme is used (Table 2). It is likely the purified recombinant H6H loses activity faster in an *in vitro* condition than in an *in vivo* cellular environment. This effect can be attributed to the accumulation of intermediate if the H6H supply is limited. Using whole cells as biocatalyst therefore significantly boosts the epoxidation activity of *BsH6H* and the total yield of scopolamine.

Conclusions

Herein, we have disclosed a new highly potent *B. sanguinea* version of hyoscyamine 6 β -hydroxylase, one of the rate-limiting bi-functional key enzymes in the biosynthesis of scopolamine. Recombinant expression of a SUMO-fused *BsH6H* in *E. coli* produces a stable fusion enzyme with increased epoxidation activity compared to other known species (10 times bigger V_{max} for 2 compared to *AbtH6H*). Truncation of the unstructured C-terminal region (30 a.a.) does not negatively influence stability nor activity of H6H. Furthermore, fermentation using whole cells results in a high scopolamine/6 β -hydroxyhyoscyamine ratio with complete transformation to scopolamine in about 28 h, therefore presenting a promising approach for the *in vivo* production of scopolamine.

Experimental section

General

(–)-Hyoscyamine hydrobromide and scopolamine were purchased from TCI America (USA), 6 β -hydroxy hyoscyamine from LKT Laboratories Inc. (USA) and catalase from Sigma Aldrich (cat. no. C9322, USA). *BsH6H* was expressed as SUMO-fusion with an N-terminal His₆-tag, subsequently removed by SUMO protease, giving the tag-free enzyme. All solvents were used as received unless otherwise specified. The mass of *BsH6H* was determined on an Agilent 6220 oaTOF spectrometer. Analytical high performance liquid chromatography (HPLC) was performed on a Mandel, equipped with a dual wavelength detector, a manual 1 ml Rheodyne manual injector and a Phenomenex Luna C18(2) column (5 μm , 4.6 $\times$ 250 mm). All HPLC solvents were filtered with a Millipore filtration system before use. LC-MS was performed on an Agilent

Table 1 Kinetic parameters for H6H variants

	K_m (μM) for 1	K_m (μM) for 2	V_{max} ($\text{nmol s}^{-1} \text{mg}^{-1}$) for 1	V_{max} ($\text{nmol s}^{-1} \text{mg}^{-1}$) for 2	Lit.
<i>BsH6H</i> (SUMO)	69	62	0.8	0.02	—
<i>BsH6H</i>	65	70	1.0	0.03	—
<i>AbtH6H</i> (His)	52	84	1.2	0.003	17
<i>AbtH6H</i>	50	93	1.5	0.004	—
<i>HnH6H</i> (His)	35	ND	3.3	ND	5
<i>AtH6H</i> (His)	15	17	11.1	4.2	24
<i>DmH6H</i> (His)	50	ND	4.6	ND	25

Table 2 Biotransformation of hyoscyamine **1** using purified enzyme and whole cells

Entry	Time (h)	1 (mg L ⁻¹)	2 (mg L ⁻¹)	3 (mg L ⁻¹)	Ratio (3/2)
BsH6H (SUMO)	4	53	42	2	0.05
BsH6H (SUMO)	24	0	74	25	0.34
BsH6H whole cells	4	5	75	19	0.25
BsH6H whole cells	24	0	7	92	13.1

Technologies 6130 LCMS using a core-shell C18-column (1.7 μ m, 100 Å, Phenomenex Kintex).

Isolation of H6HcDNA from *Brugmansia sanguinea*

We obtained *B. sanguinea* seeds from Prof. Mark Cushman (Purdue University, USA). To germinate, seeds were soaked in water for one day, sowed into pots and grown in high humidity for 4 weeks. The germinated plants were grown in a growth chamber at 22/17 °C and for 16/8 h of day/night cycle. Immature healthy roots (0.1 g) were shock-frozen in liquid nitrogen and prepared for total RNA extraction using the Trizole (Invitrogen). First strand cDNA was synthesized from 1 μ g of total RNA using M-MuLV reverse transcriptase (NEB) following the manufacture's protocol. To isolate the H6H homologue, we designed primers (F: 5'-ATGGCTACTTTTGTGTCGAAGTGGTCTAC-3' and R: 5'-TTAGACATTGATTTTATATGGCTTAACACCAG-3') from a reported H6H homologue sequence (*B. candida*)¹⁸ and amplified *BsH6H* with high fidelity Ex-Taq polymerase (TAKARA) from the cDNA. The amplified PCR products were cloned into a pGEMT vector (Promega, A3600) using blunt/TA ligase master mix (NEB, M0367). The sequences of the cloned plasmids were analyzed by sanger sequencing and the final *BsH6H* sequence is reported in the ESI.†

Cloning and heterologous expression of *BsH6H* in *E. coli*

DNA sequences encoding *BsH6H* and *AbtH6H* were *in vitro* synthesized from BioBasic Inc. (Ontario, Canada). The codon usage of these genes had been optimized for expression in *E. coli*. The genes were cloned into the pET SUMO expression vector (Invitrogen). The resulting plasmids were sequenced to ensure that *BsH6H* and *AbtH6H* were in frame with the His-tagged SUMO fusion protein. The plasmids were transformed into *E. coli* BL21(DE3). For overexpression of each of the gene, 22 ml of a fresh overnight culture of *E. coli* containing the recombinant plasmid was added to 1 L of Luria-Bertani (LB) (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl) at 37 °C with shaking (225 rpm) and cells were grown to an optical density (OD₆₀₀) of 0.6–0.8 using kanamycin as selective pressure. After putting on ice for 10 min, isopropyl β -D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM, and culture growth was continued overnight at 20 °C and shaking (225 rpm) for 15 h. Cells were harvested by centrifugation at 7000 $\times$ g for 30 min at 4 °C.

Purification of *BsH6H*

All purification steps were performed at 0–4 °C. Approximately 5 g of cell pellet was suspended in 30 ml of buffer A (25 mM NaCl, 10 mM TrisHCl and 5 mM imidazole, pH 7.6) with lysozyme (0.1 mg mL⁻¹) and DNase I (2 μ g mL⁻¹). After incubation for 15 min at room temperature followed by 20 min on ice, the cells were lysed by sonication. The cellular debris was removed by centrifugation at 30 000 $\times$ g for 30 min at 4 °C, and the supernatant was loaded onto 1 ml of a Ni-NTA resin (Qiagen). Initially 10 ml of buffer A were used to wash out contaminant proteins. The target protein was eluted by buffer A with increased imidazole concentration (25, 40, 60, 80, 100 and 200 mM imidazole, 5 ml each). The fractions were analyzed by SDS-PAGE. The fractions containing pure H6H were collected, dialyzed against Tris buffer B (25 mM NaCl and 10 mM TrisHCl, pH 7.6), concentrated using Amicon centrifugal filter units with a MWCO of 30 kDa and stored with 20% glycerol at –80 °C. For the cleavage of the SUMO tag 10 mg of SUMO-fusion protein was dissolved in 5 ml of buffer B, 0.5 ml of SUMO buffer (10 $\times$, 500 mM TrisHCl pH 8.0, 2% Igepal, 10 mM DDT) and 500 mM NaCl solution to a final concentration of 150 mM and 20 μ l SUMO protease (100 U μ L⁻¹, McLab, USA) was added and the mixture is gently shaken at 4 °C until completeness of the cleavage. Imidazole was added to the cleavage cocktail to a final concentration of 20 mM and 0.5 ml of Ni-NTA resin (Qiagen) was added. The cocktail was shaken for 1 h at 50 rpm at 4 °C and subsequently added on a fritted column. The flow-through contains cleaved H6H enzyme, which is concentrated using Amicon centrifugal filter units with a MWCO of 30 kDa and stored with 20% glycerol at –80 °C. Protein concentrations were determined using direct UV absorbance at 280 nm. Average yield was approximately 18 mg of tag-free enzyme.

Enzyme activity assay

The hydroxylase and epoxidase activity were assayed by measuring the formation of anisodamine and scopolamine respectively. The assay mixture contained: 50 mM TrisHCl buffer (pH 7.6), 0.4 mM FeSO₄, 4 mM sodium ascorbate, 1 mM 2-oxoglutaric acid, 0.2 mM (–)-hyoscyamine hydrobromide or 6 β -hydroxyhyoscyamine, 2 mg mL⁻¹ of catalase and *BsH6H*. In a final volume of 10 ml, the enzyme was incubated at 34 °C for 1 h, then quenched with 170 μ l of saturated Na₂CO₃ solution raising the pH to ~9.5, followed

by extraction with 20 ml EtOAc and drying over Na₂SO₄. After removal of EtOAc under reduced pressure, the residue was dissolved in 0.5 ml of diluted HCl (20 mM). The quantification of the alkaloids was performed by HPLC analysis.

For determination of K_m and V_{max} of both reaction steps concentration of (–)-hyoscyamine and 6β-hydroxyhyoscyamine in the above described assay were varied (0.01, 0.02, 0.04, 0.08, 0.15 and 0.25 mM) while keeping the amount of BsH6H constant (0.6 μM) and incubation time was set to 1 h. H6H activities for 1 and 2 were linear during the 1 hour assay time. Quantification of alkaloids was done by LC-MS.

HPLC method

The separation of alkaloids was monitored by UV absorbance at 210 nm. The mobile phase contained solvent A, water with 0.3% phosphoric acid (adjusted pH 7.4 by addition of triethylamine), and solvent B, MeOH. The baseline separation was achieved using a gradient method described earlier.

Conflicts of interest

The authors declare no competing interest.

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